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Appendix 1

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TITLE: Optimization of Fibroblast Growth Factor-1 as an Anabolic Agent for Osteoporosis

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13. ABSTRACT ***(Maximum 200 Words)***

The long term goal of this research program is to develop fibroblast growth factor-1 through rational protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. The specific aims of this research plan are:

1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells in vitro, on bone formation in vivo and to assess their toxicological or undesirable effects.
2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicity.

Significant progress was made towards these goals during the course of support for this program. A number of different FGF-1 variants were generated and their in vitro and in vivo efficacy tested. Among these Arg136Lys mutant was the most osteoinductive followed by Cys-free > FGF-1 > FGF-HBGAM chimera. Furthermore, we demonstrated that injection of FGF-1 directly into the marrow cavity induces new bone formation suggesting the possibility of local delivery as a strategy to specifically increase the density of bone that are at risk of fracture. Finally, we can show that FGF can enhance the osteogenic differentiation of marrow stromal cells suspended in fibrin sealant, which serves as a manipulable delivery vehicle for both cells and growth factor(s).

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Introduction

The long-term goal of this research has been to develop FGF-1 through protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. Osteoporosis afflicts nearly 200 million people worldwide, and this number will increase in the foreseeable future as the population ages. It is likely that all people with the disease will benefit from treatments designed to increase bone mass. The greatest therapeutic challenge in the osteoporosis field at the present time is the identification of agents that promote significant new bone formation. Although there are effective resorption inhibitors for the treatment of osteoporosis (bisphosphanates, estrogens, and calcitonin), these drugs essentially stabilize bone mass but do not lead to substantial increases in bone mass or the restoration of trabecular bone microarchitecture. For patients with severe and established osteoporosis, there is a tremendous need for therapeutic agents that stimulate bone formation and initiate the cascade of events involved in osteoblast differentiation. Those agents that are known to have a stimulatory effect on new bone formation are fluoride, low-dose intermittent parathyroid hormone and its analogs, and the peptide growth factors that are incorporated into the bone matrix and released from bone as it is resorbed.

During the past several years, it has become apparent that members of the FGF family of growth factors and their receptors are essential for normal skeletal growth (1). The preliminary data that formed the basis of the original application demonstrated a significant osteogenic potential for local and systemic administration of FGF-1 *in vivo*. The data also documented certain toxicological or undesirable effects associated with these treatments. Together these data

indicated that the therapeutic window is relatively narrow. In the progress report for the first year of funding, Dr. Burgess outlined his progress on the generation of several mutants of FGF-1 and chimeric proteins. He also reported on the *in vitro* and *in vivo* activities of these proteins. Those studies suggested that further evaluation of existing mutants and production of additional mutants or chimeric proteins may improve the efficacy of FGF-1 as an anabolic factor in the treatment of osteoporosis. In subsequent years, the animal model, route and duration of treatment with FGFs was refined and the toxic / undesired effect of FGF on other organs, particularly the kidney was explored. Since taking responsibility for this program in June of 2000, I presented data demonstrating that among the FGF-1 variants tested, the Agr136Lys mutant of FGF-1 was most osteoinductive followed in order by Cys-free, wild type FGF-1, and FGF-HBGAM. In addition, the osteoinductive potential of locally delivered FGF-1, and the potential of the local delivery of FGF-1 and osteoblast precursor, for example for fracture repair was explored.

The original specific aims of the proposal were:

1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo* and to assess their toxicological or undesirable effects.
2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

Body

Year 1 accomplishments: A critical accomplishment of year 1 was to optimize FGF-1 purification scheme. This was accomplished by the finding that bacterial produced FGFs from inclusion bodies could be solubilized in 6M urea, bound to heparin in the presence of urea followed by slow removal / renaturation of the protein with a 6M to 0M urea gradient. The

renatured proteins were then eluted with a NaCl gradient. This simple, but important modification allowed purification of tens of milligrams per liter of culture for each FGF construct which allowed large scale in vivo studies to be undertaken. During Year 1, Dr. Burgess also generated the following FGF-1 variants as already described in his Year 1 Progress Report:

1) Cys-free – each of three cysteines mutated to serines
2) S130K – serine at position 130 mutated to lysine
3) S31K – serine at position 31 changed to lysine
4) FGF-HBGAM chimera – fusion of HBGAM to the amino terminus of FGF-1
5) HAFGF-1 – nine residue hemagglutinin epitope fused to the C-terminus of FGF-1

The rationale for the design of each of these constructs was detailed in progress report for Year 1 (copy enclosed for reference). In addition, the result of an in vivo experiment administering wild type and cys-free FGF-1 to overiectomized rats was described. It was reported that new bone formation could be documented histologically for rats receiving 200 micro-g/kg of FGF-1, 200 micro-g/kg cys-free and 20 micro-g/kg cys-free but no histological evidence of new bone formation was noticed for rats receiving 20 micro-g/kg of FGF-1. Interestingly, all four treatment groups showed the same increase in bone strength compared to untreated controls in a bending assay (please refer to Year 1 Progress Report for details).

Year 2 accomplishments: In year 2, the in vivo overiectomy model was modified to more accurately reflect established osteoporosis. Instead of treating rats with various FGF constructs immediately after overiectomy, the animals were maintained for 4.5 months following overiectomy (animals stabilize at a new, albeit lower, bone mass) prior to FGF treatment. Again, it was shown that bone mass could be restored with 200 micro-g/kg FGF-1, as well as 200 or 20 micro-g/kg cys-free FGF-1. Treatment with 20 micro-g/kg FGF-1 was histologically ineffective,

as before. The conclusion was made that cys-free FGF-1 is ten times more potent than wild type-FGF-1.

Potential toxic effects of FGF-1 and csy-free FGF-1 on the kidney were qualitatively evaluated. Both forms of FGF cause expansion of the glomerular basement membrane, but it was felt that cys-free was better tolerated than wild type FGF-1 as detailed in Year 2 Progress report (copy enclosed for reference).

Year 3 accomplishments: In year 3, the effects of systemic injections of FGF-1, cys-free, S130K FGF-1 and HBGAM-FGF-1 were evaluated in rats that were aged for five months following overiectomy. Based on quantitation of mineralized tissue under the growth plate of tibias from these animals it was concluded that all were anabolic with S130K mutant producing most bone followed by cys-free, HBGAM-FGF-1 chimera and FGF-1. Details of these findings were reported in the Year 3 Progress Report which is included for further reference.

In addition, in vitro differentiation of marrow stromal cells to osteoblasts was established and the effects of FGF-1 and its variant was assessed. It was demonstrated that FGF-1 and HBGAM-FGF-1 enhance the osteoblastic phenotype, i.e. mineralization of the cultures. Interestingly, the cys-free mutant inhibited mineralization in vitro. Consistent with its enhanced mitogenic effect compared to wild type FGF-1, it was concluded that the cyc-free maintained cell proliferation and prevented them from exiting the cell cycle to initiate differentiation.

Year 4 accomplishments: This grant was transferred from Dr. Burgess to me in June of 2000 due Dr. Burgess' resignation from the ARC earlier that year. Realizing the potential hurdles still to overcome by systemic administration of FGF, I focused on localized administration of the growth factor.

One new FGF-1 construct, R136K FGF-1, was produced during year 4. The rationale behind this construct was as follows. We were interested in testing the bone anabolic efficacy of delivering FGF-1 locally by incorporating the growth factor in fibrin sealant (a mixture of fibrinogen and thrombin produced and marketed by the American Red Cross). We wished to minimize degradation of FGF-1 by thrombin, a protease that is required for clotting of fibrinogen and therefore a necessary component of the fibrin sealant delivery. Wild type FGF-1 is cleaved by thrombin at Arg 136, and ordinarily we add heparin to protect the FGF from thrombin cleavage. Although heparin renders FGF very resistant to thrombin cleavage, it inhibits mineralization by osteoblast cultures in vitro, and causes osteoporosis in vivo. For this reason, the thrombin cleavage site of FGF-1, Arg 136 was mutated to a Lys. We showed that R136K mutant is more thrombin resistant as detailed in our last Progress Report which is enclosed for reference. We also established that the R136K FGF-1 has identical mitogenic activity to the wild type protein (please refer to last year's progress report for details).

Localized bone induction. The basic protocol has been to present FGF, or various mutant forms of it in a slow release form juxtaposed to calvarial bones for two weeks, followed by histologic assessment of bone formation. More specifically, we mix 1-5 μg of each FGF variant in 25 μl of water with 50 μl of 400 mg/ml fibrinogen, 24 μl of 5 U/ml thrombin, and 1 μl of 10,000 U/ml heparin in a 1 ml syringe. The fibrinogen / thrombin mixture carrier was developed by the American Red Cross and is marketed as ARC "Fibrin Sealant". Previous studies have shown that the fibrin sealant can be formulated to release trapped compounds, e.g. FGF-1, for up to 30 days. The fibrin / FGF-1 mixtures are incubated at room temperature for 2 hours to allow the fibrinogen to be cleaved by thrombin and form a firm gel. Mice are anesthetized with a mixture of xylazine and ketamine, the skin over the calvaria is opened and the preformed plug of fibrin

sealant or fibrin sealant containing each FGF variant is placed over the calvaria. Subsequently, the skin is closed with wound clips. Two weeks later, the animals are sacrificed, the calvaria dissected and prepared for histology. Using this assay we compared the bone inductive affects of wild type FGF-1, Cys-free FGF-1, FGF-HBGAM chimera and R136K FGF-1. From these experiments we showed that the R136K mutant is the most osteoinductive followed by Cys-free > FGF-1 > FGF-HBGAM chimera. We presume that the R136K mutant is more potent than the wild type FGF-1 due to its resistance to protease degradation.

A second series of experiments were designed to test the ability of FGF-1 to promote new bone formation directly in the intramedulary cavity. In this case, FGF-1 was mixed with fibrin sealant, as described above, except that the mixture was injected immediately, prior to forming a gel, into the marrow space of anesthetized rat tibias. The injection needle entered the marrow directly through the skin and under the kneecap. We confirmed that the needle had entered the marrow cavity by X-ray. Two weeks later the tibias of the rats were processed for histology and the results showed new bone formation in the marrow cavity. These results illustrated the feasibility of administering FGF directly into bones that are at risk of fracturing.

Key Research Accomplishments

- Produced FGF-1 in bacteria, and optimized purification and renaturation procedures to recover tens of milligram quantities of the growth factor for long term in vivo studies.
- Produced six variants of FGF-1: cys-free, S130K, S31K, S136K, FGF-HBGAM chimera and HAFGF-1.
- Demonstrated the efficacy of FGF-1, cys-free, S130K and FGF-HBGAM chimera to restore bone mass in aged overiectomized rats by intravenous injection of the growth factors.

- Established two new models for localized bone induction, specifically administration of FGFs in fibrin sealant over the calvaria and directly into the marrow cavity.
- Established osteoblast migration and attachment assays.
- Established stem cell differentiation assays.
- Established histological analysis of mineralized sections.
- Demonstrated reduced hypotensive activity of cys-free mutant (previous years).
- Demonstrate reduced toxicity of cys-free FGF-1 on the kidney.

Reportable Outcomes

- Tissue Engineering/Regenerative Healing/Stem Cell Biology Conference
  Cambridge Healthtech Institute, Pittsburgh, Pennsylvania
  Title: Fibroblast Growth Factor-1: Multiple Aspects of Bone Formation.
- D.J. Mackenzie, R. Sipe, D. Buck, W. Burgess, J. Hollinger. "Recombinant Human Acid Fibroblast Growth Factor and Fibrin Carrier Regenerate Bone." Plast. Reconstr. Surg. 107:989-96. (1999).
- Dunstan, CR, Boyce, R, Boyce, BF, Garrett, IR, Izbicka, E, Burgess, WH, Mundy, GR. "Systemic administration of acidic fibroblast growth factor (FGF-1) prevents bone loss and increases new bone formation in overiectomized rats." J. Bone and Mineral Res. 14:953-9.

Conclusions

Together, the data summarized in the body of this report and presented in previous progress reports demonstrate that the original hypothesis and statement of work were of merit; although since taking responsibility of this grant in June of 2000, I have focused on localized use of FGF-

1. We have directly tested the bone anabolic effects of all currently available FGF-1 variants. The challenge for the future will be to further reduce the toxic effects of FGFs on the kidney, although successful anabolic effect following local administration may alleviate the kidney epithelial proliferation in response to the growth factor.

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The long term goal of this research program is to develop fibroblast growth factor-1 through rational protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. The specific aims of this research plan are:

1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo* and to assess their toxicological or undesirable effects.

2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects. During the current year of support we have made significant progress with regard to these specific aims. The data obtained to date indicate that the proposed research plan was realistic and rational. We have generated five different FGF-1 variants and shown utility of some of these variants *in vivo*. The results of the *in vivo* and *in vitro* studies indicate a logical progression towards optimizing the anabolic activity of the protein for bone will be possible. The cys-free mutant of FGF-1 has been studied extensively and this construct is likely to become the parent protein to which other mutations will be incorporated.

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Wilson R. Burgess 101697

PI - Signature Date

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INTRODUCTION

The long term goal of this research program is to develop fibroblast growth factor-1 via protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. Osteoporosis is a disease which afflicts nearly 200 million people worldwide and this number is expected to double in the next 25 to 35 years. It is probable that all people with the disease will ultimately benefit from treatments to increase bone formation. A more acute and relevant need for an anabolic treatment is a prophylactic for the approximate 700,000 hip fractures each year in the United States, Europe and Japan combined. The greatest therapeutic challenge in the osteoporosis field at the present time is identification of an agent that promotes significant bone formation. Although there are effective resorption inhibitors for osteoporosis (bisphosphonates, estrogen and calcitonin), these drugs essentially stabilize bone mass and do not cause substantial increases in bone mass or restore trabecular bone microarchitecture. For patients with severe and established osteoporosis, there is thus a need for therapeutic agents which stimulate bone formation and initiate the cascade of events involved in osteoblast differentiation. Those agents which are currently known to have a stimulatory effect on new bone formation are fluoride, low-dose intermittent parathyroid hormone and its analogs, and the peptide growth factors which are incorporated into the bone matrix and released from bone as it resorbs.

During the past several years, it has become apparent that members of the fibroblast growth factor (FGF) family of ligands and receptors are essential for normal skeletal growth (Rousseau, et al., 1994). Recent observations demonstrate that a variety of inherited disorders of skeletal growth are due to point mutations in FGF receptors. It is also known that members of the FGF family are expressed by bone cells, stored in the bone matrix and stimulate bone formation *in vivo*. Our preliminary data indicate a significant osteogenic potential of systemic administration of FGF-1 in animal models of osteoporosis. There are also certain toxicological or undesirable affects associated with these treatments. The results obtained to date indicate that the therapeutic window is relatively narrow. We have generated a variety of mutant forms of FGF-1 that appear to be better candidates for the proposed therapeutic uses. We believe that a further evaluation of existing mutants and production of additional mutants or chimeric proteins will improve the efficacy of the FGF-1 derivatives as anabolic factors in the treatment of osteoporosis and other disorders of bone metabolism.

The original specific aims of the proposal were:

1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo* and to assess their toxicological or undesirable effects.

2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

Progress towards these aims achieved during the first year of funding are summarized below.

BODY

Based on the original "statement of work" progress during the first year was excellent. The animal models and histomorphometric assays have been established at the Holland Laboratory, large scale production of specific FGF-1 mutants and chimeric proteins have been completed and tested *in vitro* and *in vivo*. Based on these data, second generation molecules have been identified that will be tested in the coming year. A summary of these results is provided below.

A major problem in the large scale production of several of the FGF-1 mutants in prokaryotes was the fact that the majority (>95%) of the recombinant protein ended up in the inclusion bodies of the bacteria. The protein could be solubilized with urea, but could not be purified in an active state following simple dialysis. We found that the mutant FGF-1s maintained a relatively high affinity for immobilized heparin even in the presence of 6M urea. The solubilized proteins were adsorbed to heparin-Sepharose in the presence of urea, then a reverse gradient of 6M and 0M urea buffers were passed through the column. Biologically active protein could then be eluted with a normal NaCl gradient. We infer that the bound heparin allows the protein to refold in an active conformation as the urea is slowly removed during the first gradient step. We can now produce 50-100 mg quantities of all the recombinant proteins we have constructed from a single prep.

The mutant or chimeric forms of FGF-1 produced to date are:

1) cys-free - each cysteine residue (3) changed to serine.
2) S330K - the serine at position 130 changed to a lysine residue.
3) S31K - the serine at position 31 changed to a lysine residue.
4) HBGAM chimera - a fusion of the protein HBGAM (also known as bone specific protein 1) to the amino terminus of FGF-1.
5) HAFGF-1 - the 9 residue hemagglutinin epitope fused to the carboxy terminus of FGF-1.

The rational and the status of each of these constructs are detailed below:

1) cys-free: studies of the cys-free mutant were a major focus of the original application and represents the FGF-1 variant that is farthest along at this point. It is likely that the future mutants will utilize the cys-free construct as the parent factor. We have completed 1 *in vivo* study comparing the cys-free mutant to wild-type FGF-1 in the ovariectomized (OVX) rat model of osteoporosis. The preliminary data on systemic FGF-1 in this model in the original application involved daily treatment for 28 days. In the present study we used a more realistic regiment of 28 week-day injections over a total of 38 days. The study utilized 200 or 20 μg/kg of wild-type and cys-free FGF-1. The injections were started two weeks after ovariectomy. At the conclusion of the study the rats were euthanized and femurs, tibia, vertebrae, spleen, kidney, liver and thymus retrieved for study. Histological analysis of the vertebrae and tibia by H&E staining of sections of demineralized tissue revealed a dramatic loss of bone mass in the untreated animals. The bone mass of both 200 μg/kg groups was normal. There was a noticeable loss of bone mass in the 20 μg/kg wild-type treated animals that was not apparent in the cys-free group. These

results appeared consistent with the *in vitro* and local *in vivo* studies presented in the original proposal. We also examined the strength of the femurs of these animals in a 3 point bending assay. It should be noted that such functional assays were not a part of the original application. We perceived this to be a weakness of the project. Though a collaboration with a mechanical engineering Ph.D. student from the University of Maryland, Baltimore County, we were able to establish this study. The results are summarized in Figure 1 (appendix). The data represents the force in pounds required to break the femurs of animals from the different treatment groups. It can be seen that the OVX animals exhibit ~75% reduction in bone strength when compared to normal rats. It is also clear that the 200 or 20 μg/kg treatments of both wild-type and cys-free FGF-1 were able to maintain (or establish) normal bone density in the OVX animals. This result was not anticipated based on the histological analysis, but presents a pleasant surprise. An identical study is presently in progress using animals that were aged for three months following ovariectomy. This study will provide a clear analysis of the relative efficacies of the two doses of the two proteins to restore bone mass and density.

2) S130K: Studies of this mutant have not yet been conducted *in vivo*. *In vitro*, this mutant is more specific for target cells that correlate with *in vivo* performance. Our strategy in the next year is to incorporate this mutation into the cys-free construct.

3) S31K: Studies of this mutant are also limited to *in vitro* assays. We have a manuscript in preparation (will be sent at time of submission) that demonstrates increased affinity of this protein for immobilized heparin with no change in mitogenic activity. We believe that increased heparin affinity will improved the targeting of the protein to bone in local and perhaps systemic administration. This hypothesis remains to be tested.

4) HBGAM chimera: Studies of this construct have included *in vitro* and local *in vivo* experiments. The rationale for the construction of this chimeric protein was based on the reports that HBGAM is found at relatively high concentrations in bone (Gieffers, et al. 1993) and that osteoblasts have a relatively high number of cell surface receptors for the protein. Our *in vitro* data demonstrates that the chimeric protein is relatively independent of exogenous heparin for maximal mitogenic activity when compared to wild-type FGF-1 (Hampton, et al. manuscript in preparation). Local injections of the chimera over the calvaria of mice result in new bone formation similar to that seen with FGF-1. A major goal of the next year of support is to determine the effectiveness of this construct in systemic studies and to determine whether the chimera in conjunction with the cys-free and other mutants should become the base or parent construct for future studies.

5) HAFGF-1: This construct was produced to take advantage of the high quality antibody that we have generated to the HA epitope. The antibody will have utility in quantitating the localization of HAFGF-1 to bone and in studies of the distribution of the protein following systemic administration. The use of this construct and the HA antibody for these studies will allow us to discriminate between endogenous and administered FGF-1.

CONCLUSIONS

In summary, progress on the specific aims has been good. The addition of the 3 point bending assays to the methods of approach is important. The data generated with the cys-free mutant indicates that it should become the parent compound for future mutations. We believe the data obtained to date supports our hypothesis that systemic FGF-1 can become an effective therapeutic agent for this treatment and prevention of osteoporosis. We look forward to continued support and continued progress in this critical area of research.

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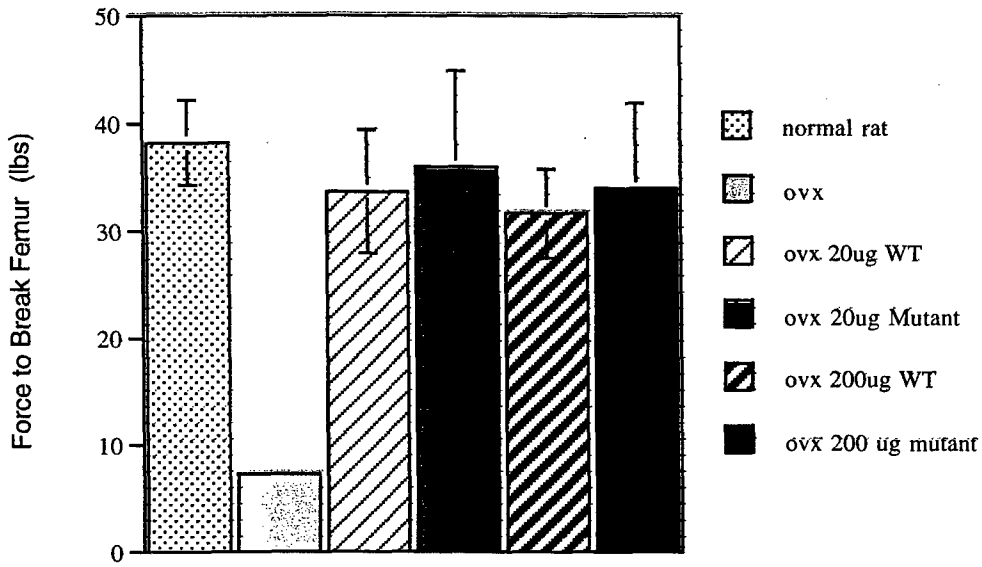
Force to Break Femur (lbs)
50
40
30
20
10
0
normal rat
ovx
ovx 20ug WT
ovx 20ug Mutant
ovx 200ug WT
ovx 200 ug mutant

AD Appendix 2

GRANT NUMBER DAMD17-96-1-6314

TITLE: Optimization of Fibroblast Growth Factor-1 as an Anabolic Agent for Osteoporosis

PRINCIPAL INVESTIGATOR: Wilson H. Burgess, Ph.D.

CONTRACTING ORGANIZATION: American Red Cross
Holland Laboratory
Rockville, Maryland 20855

REPORT DATE: October 1998

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Wilson H. Burgess, Ph.D.

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13. ABSTRACT *(Maximum 200*

The long term goal of this research program is to develop fibroblast growth factor-1 (FGF-1) through rational protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. The specific aims of this research plan remain:

1) to evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo*, and to assess their toxicological or undesirable activities
2) to generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

During the current year of support we have made significant progress with regard to these specific aims. The most important find was the demonstration that systemic FGF-1 could not only preserve but restore bone mass in animal models of osteoporosis. In addition, we were able to establish the fact that site-directed mutagenesis of FGF-1 could be used to generate mutant forms of the protein with enhanced anabolic activity for bone formation and reduced toxicological effects. This is the first demonstration of the concept that formed the basis of the original specific aims.

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	16. PRICE CODE

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Wilbur H. Burgess

PI - Signature Date

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Introduction

The long term goal of this research program remains to develop fibroblast growth factor-1 (FGF-1) via protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. Osteoporosis is a disease which afflicts nearly 200 million people worldwide and this number is expected to double in the next 20-30 years. It is likely that all people with the disease would benefit from treatments to increase bone mass. The greatest therapeutic challenge in the osteoporosis field at the present time is the identification of an agent that promotes significant new bone formation. Although there are effective resorption inhibitors for the treatment of osteoporosis (bisphosphanates, estrogen, and calcitonin) these drugs essentially stabilize bone mass but do not lead to substantial increases in bone mass or the restoration of trabecular bone microarchitecture. For patients with severe and established osteoporosis, there is a tremendous need for therapeutic agents that stimulate bone formation and initiate the cascade of events involved in osteoblast differentiation. Those agents that are known to have a stimulatory effect on new bone formation are fluoride, low-dose intermittent parathyroid hormone and its analogs, and the peptide growth factors that are incorporated into the bone matrix and released from bone as it resorbs.

During the past several years, it has become apparent that members of the FGF family of ligands and receptors are essential for normal skeletal growth (Li *et al.*, 1997). The preliminary data that formed the basis of the original application demonstrated a significant osteogenic potential for local and systemic administration of FGF-1 *in vivo*. The data also documented certain toxicological or undesirable effects associated with these treatments. Together these data indicated that the therapeutic window is relatively narrow. In the progress report for the first year of funding, we outlined our progress on the generation of several mutants of FGF-1 and chimeric proteins. We also reported on the *in vitro* and *in vivo* activities of these proteins. The studies confirmed that a further evaluation of existing mutants and production of additional mutants or chimeric proteins may improve the efficacy of FGF-1 as an anabolic factor in the treatment of osteoporosis.

The original specific aims of the proposal were:

1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo* and to assess their toxicological or undesirable effects.
2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

Progress towards these aims achieved during the previous and current funding period are summarized in the following report.

Body

In the progress report for the first year of support we described the solubility problems we encountered with a variety of the mutant FGF-1 constructs (protein ended up in inclusion bodies of bacteria). Although a solution to the purification of these proteins was provided, we have observed variability in the biological activities of the urea solubilized proteins. Although this is not a critical problem to the long term goals of the study, it is an existing problem that impacts the progress of the work. The take home message is that even for previously characterized mutants of FGF-1, the *in vitro* biological activity of the proteins must be validated prior to systemic or other *in vivo* assays. Despite this inconvenience, significant progress has been made during the second year of support. This variability in the specific activity of the recombinant proteins is the only significant problem encountered during this year of support.

The primary focus of the current year has been on the cys-free mutant of FGF-1. During the first year of support, we established that this mutant FGF-1 was ~10 fold more potent than the wild-type protein in maintaining trabecular bone mass in ovariectomized rats. Although these findings were encouraging, they did not address directly the heart of the real problem which is restoration of bone mass. Our approach to this problem has been to delay administration of FGF-1 or FGF-1 mutants until three months post-ovariectomy, at which time, significant loss of trabecular bone has occurred. This model for restoration of bone mass has occurred. This model for restoration of bone mass has been the focus of all of the experiments conducted during this funding period. There are

serious concerns regarding long term systemic administration of any polypeptide growth factor. We believe that systemic FGF-1 treatment will be relatively brief to restore bone mass. Once the increased bone mass is established, current therapies to maintain bone mass should be effective in management of the disease.

We have also begun a detailed analysis of the effects of systemic FGF-1 on other organs or tissues. The gross analysis (appearance, wet weight) did not indicate significant pathology associated with the treatments. A more detailed examination of various organs revealed potentially adverse effects on the kidney (see below). A second advance in our analysis of the *in vivo* data relates to quantitation of the restoration of bone mass in the ovariectomized animals. We were able to purchase (with institutional funds) a Bioquant image analysis hardware and software package. One of the members of my laboratory, Lawyna Holland, is the primary operator of the system. She is currently analyzing all of our H and E stained tissue sections from previous systemic studies in order to quantitate the bone mass in the different treatment groups. It should be noted that our ability to conduct significant histomorphometric analysis of our studies was one of the few concerns raised in the initial peer review of our application. In summary to this general introduction, we believe we have maintained or surpassed the original statement of work for the first two years. We have encountered several problems and have solutions in place. We have also improved our ability to analyze existing data and have begun a detailed analysis of the toxicology of the mutants that appear to be the most effective anabolics for bone. Although it is beyond the scope of a progress report to summarize all of the data, documentation of what we consider to be the most significant findings is provided below.

Systemic Treatment of Aged Ovariectomized Rats: The major challenge in the treatment of osteoporosis is the restoration of lost bone mass. In the previous progress report, we demonstrated that FGF-1 would effectively prevent the loss of bone mass following ovariectomy. In the current year, we have allowed animals to lose bone mass by delaying systemic treatment until 4.5 months following ovariectomy. The results of some of these studies are provided on appendices 1-4. At 4.5 months, animals were given 200μg/kg

FGF-1 by tail vein injection for 35 days on a 5 days on, 2 days off dosing. Two weeks after the last injection animals were euthanized and tissue was analyzed. The figures show H and E or toludine blue staining of frontal sections through the tibias of these animals.

Appendix 1. H and E stain of normal rat, rat 4.5 months after ovariectomy (OVX) and an ovariectomized rat given FGF-1 starting at 4.5 months. The dramatic loss of trabecular bone is apparent in the OVX animal. A break can be seen in the cortical bone that occurs frequently during sectioning of tissue from these animals. In contrast, 5 weeks of systemic FGF-1 restores significant mass to the trabecular bone of these animals. This loss and restoration is more apparent in the higher magnification of Appendix 2. It can also be seen from this figure that the cartilage of the growth plate is normal in all the animals. This is also clear from the toludine blue stained figure in Appendix 3. Appendix 4 illustrates something that we observed for the first time this year and provides an explanation for the loss of strength in the cortical bone of the tibial shaft. The figure shows toludine blue stained sections of the remodeling stacks in the shaft. There are areas of bone resorption and formation that account for the total turnover of bone that occurs every 3 years or so. In the normal rat the bone adjacent to the cartilage stacks is cellular. In the OVX rats adjacent bone is acellular with an amorphous osteoid appearance. A dramatic increase in the cellularity and structure of bone adjacent to the stacks is apparent in the OVX animals receiving FGF-1. These date demonstrate that the anabolic effects of systemic FGF-1 are not limited to bone easily accessible from the marrow.

Cys-free Mutant of FGF-1: In the original application we provided *in vitro* data to suggest that the cys-free (3 serines changed to cysteine) mutant of FGF-1 was ~10 fold more potent as a mitogen for osteoblastic cells than the wild-type protein. During the current year of support we conducted a systemic study of the wild-type and mutant FGF-1 in the aged OVX animal model described above. We treated animals with 200 or 20μg/kg of wild-type or cys-free mutant. H and E stains of sections from the tibia of these animals are shown in Appendix 5. The figure shows the results obtained using

20μg/kg of the two proteins. At 20μg/kg the wild-type FGF-1 is not effective in restoring trabecular bone mass whereas significant trabecular bone can be seen in the cys-free treated animals. The results are seen more clearly at a higher magnification (Appendix 6). It should be noted that these were random fields selected by a technician who was blind to the study. Obviously we need to qualify the bone mass in these treatment groups. Such studies are in progress using the Bioquant system described previously.

Pathology/Toxicology: A weakness of the original application was the lack of detail regarding assessment of undesirable effects of systemic therapy on other organs. During the current year of support, we have addressed this weakness by conducting histological examinations of organs collected from the various treatment groups. A variety of unrelated experiments had established that FGF-2 (basic FGF) injections into normal mice induced renal glomerular capillary injury. Studies were conducted to determine whether systemic administration of FGF-1 induced similar changes in rats. Renal tissues were processed for electron microscopy studies. The ultrastructural changes were evaluated blindly by a collaborator, Patricio Ray, M.D., of the Children's National Medical Center at no cost to the program. The histological changes were scored on a semiquantitative scale of 0 (normal tissue) to 3 (most changes). Panels A-F of Appendix 7 show representative micrographs of the glomerular ultrastructural capillary changes observed.

A) Control rats: Normal renal glomerular capillaries and glomerular basement membranes (GBM, arrows). Score 0

B) Control ovariectomized rats: Normal renal glomerular capillaries and GBM. Score 0

C) Ovariectomized rats injected with 20μg/kg/day mutant aFGF. Slight focal increase in the GBM thickeners (arrows). Score 0.5

D) Ovariectomized rats injected with 20μg/kg/day aFGF. Slight focal increase in the GBM thickness, endothelial and mesangial matrix expansion. Focal and slight effacement of the glomerular foot processes (arrows). Score 1

E) Ovariectomized rats injected with 200μg/kg/day mutant aFGF. Slight wrinkling of the GBM and increased focal thickness of the GBM. Score 2

F) Ovariectomized rats injected with 200μg/kg/day aFGF. Folding, wrinkling and slight thickening of the GBM. Increased mesangial matrix expansion and increased interstitial space between renal cortical tubules. Score 3

Together the data obtained on restoration of bone mass and reduced toxicology/pathology of the cys-free mutant relative to wild-type protein represent the first proof of principle that FGF-1 can be engineered at the sequence level to generate a more specific and potent anabolic agent for bone. The concept that the protein could be mutated to improve its desire while reducing the undesired effects was the crux of the original application. Plans for the next year of funding will focus on quantitation of these results and the generation of additional mutants based on the cys-free or HBGAM-chimera of FGF-1.

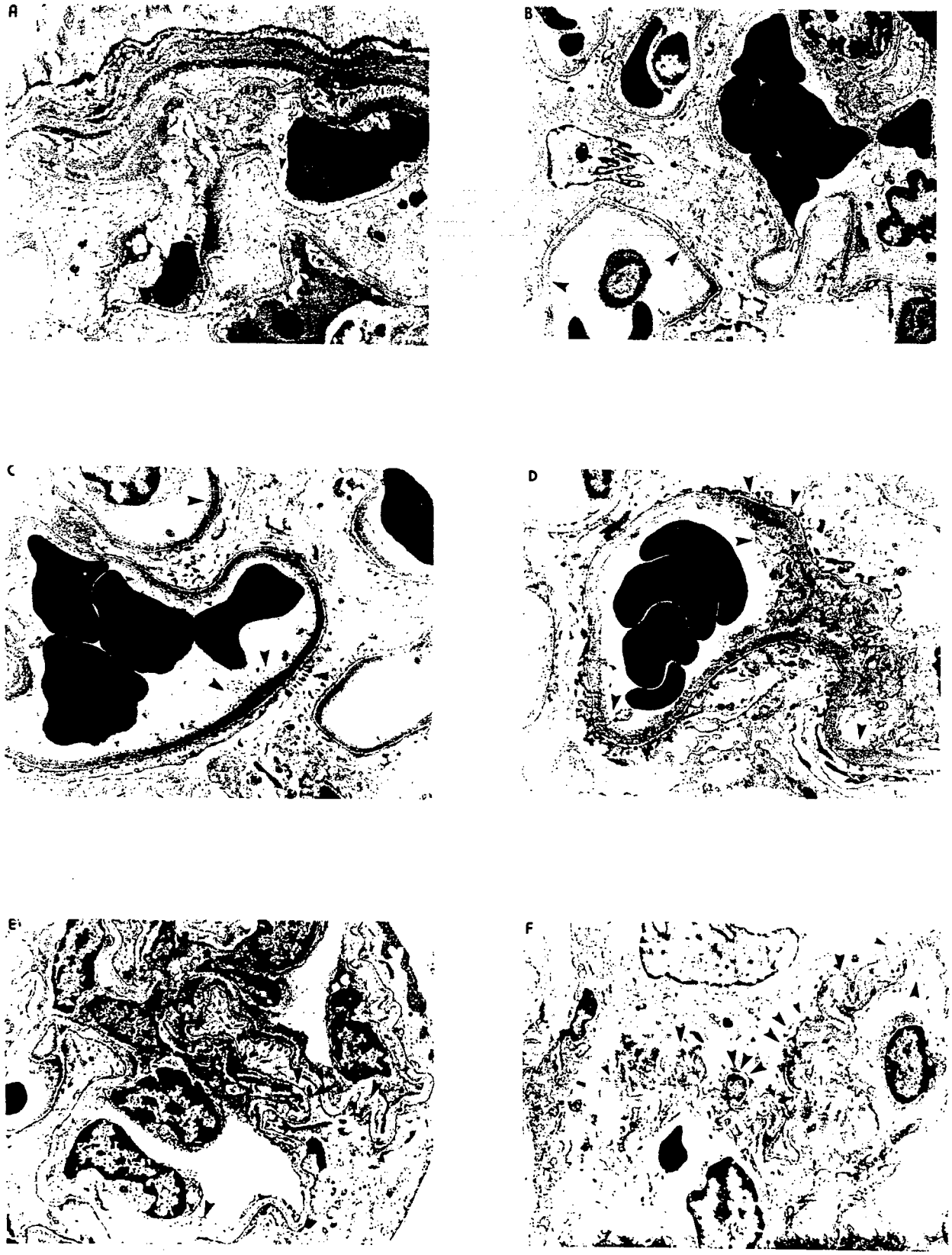
Studies on Alternative Administration of FGF-1: Two studies were conducted to evaluate methods to improve the treatment regimen to one that was more compatible with standard care. The first utilized intramuscular injection of FGF-1 in the OVX rats. These treatments were not effective in restoring bone mass and resulted in morbidity at the sites of injection. The second study evaluated a one week on/one week off treatment with FGF-1. The preliminary histological analysis of this treatment indicates that the on/off treatment is as good or better than continuous therapy. Quantitation of these studies are in progress.

Conclusions

In summary, progress towards the specific aims of the original application has been steady and consistent with or ahead of the original statement of work. The data establishing the ability of systemic FGF-1 to restore bone mass in aged OVX animals is impressive relative to any published treatment. The demonstration that site-directed mutagenesis can be used to enhance a desired activity while reducing untoward side effects is unique in the FGF-1 field. The data will benefit from the quantitative analysis

Wild-Type FGF-1

Mutant FGF-1


A
B
C
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E
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AD_____

Appendix 3

Award Number: DAMD17-96-1-6314

TITLE: Optimization of Fibroblast Growth Factor-1 As An Anabolic Agent for Osteoporosis

PRINCIPAL INVESTIGATOR: Wilson Burgess, Ph.D.

CONTRACTING ORGANIZATION: American Red Cross
Rockville, Maryland 20855

REPORT DATE: October 1999

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13. ABSTRACT ***(Maximum 200 Words)***

The long term goal of this research program is to develop fibroblast growth factor-1 (FGF-1) through rational protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. The specific aims of this research plan remain:

1) to evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo*, and to assess their toxicological or undesirable activities
2) to generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

During the current year of support we have made significant progress with regard to these specific aims. The most important finding was the identification and production of FGF-1 variants with enhanced anabolic activity for bone. We also identified differences in the abilities of these constructs to promote osteoblast differentiation from marrow cultures. Finally we have demonstrated for the first time the ability of an FGF-1 variant to exhibit increased activity for bone with reduced hypotensive effects relative to the wild-type protein.

14. SUBJECT TERMS
Osteoporosis, Fibroblast Growth Factor-1, Site-Directed Mutagenesis, Osteoblast, Differentiation, Hypotension

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William H. Burgess 10/19/99

PI - Signature Date

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Progress towards these aims achieved during the previous and current funding period are summarized in the following report.

Body

In the progress report for the previous year of support, we described our studies of the cys-free mutant of FGF-1. We demonstrated that this mutant was ~10 fold more active than wild-type FGF-1 in restoring trabecular bone mass in aged ovariectomized rats. In addition, we provided histological evidence that the cys-free mutant had reduced toxicological effects on renal glonerular capillaries and basement membranes. This represented the first data to indicate that FGF-1 could be modified through site-directed mutagenesis to exhibit enhanced anabolic activity for bone with reduced undesirable side effects. Together this data validated the hypothesis of the original application. During the current year of support we have developed several important *in vitro* assays for evaluation of different mutants, initiated blood pressure studies and evaluated several new FGF-1 constructs for their anabolic effects on bone. In addition we have established the capacity to study non-demineralized sections of bone. This allows us to make a better assessment of the quality of the new bone formation induced by systemic FGF-1 therapy in the ovariectomized rat model of osteoporosis. These results are summarized below.

New FGF-1 Constructs: Two new FGF-1 constructs were produced during the current year of support. One is a chimeric molecule consisting of the protein HB-GAM (heparin binding growth associated molecule) fused in frame with wild-type FGF-1. We and others have shown that HB-GAM is not a mitogen for a variety of cell lines (2). It has been reported to be found at relatively high concentrations in bone and to be a chemoatractant for osteoblastic cells (3). In addition over-expression of the protein in transgenic animals results in increased thickness of cortical bones. We expressed the chimeric protein with the thought that the HB-GAM portion would target FGF-1 more selectively to bone. Studies performed to date with the purified chimera are described in subsequent sections. The second construct referred to as FGF-1 678, has a serine to lysine substitution at position 130. We have shown that the region of FGF-1 is a major heparin-binding domain (4). In addition when basic residues in this region are replaced by mutagenesis

with non-basic amino acids, the protein exhibits reduced heparin affinity and reduced mitogenic activity (5). The 678 mutation represents an attempt to increase mitogenic potency through the addition of extra basic amino acids in the region.

Effects of FGF-1 Constructs on Bone Marrow Differentiation: Two six week old male SD rats were sacrificed by CO_2 asphyxiation and their femora and tibia removed and cleaned of fibrous tissue. The ends of the bones were removed and the marrow flushed out, using a syringe and 18G needle, with 5-10ml alphaMEM. The marrow cells were pelleted by centrifugation (5 min at 800G), the supernatant removed, the cells resuspended in 10ml alphaMEM then filtered through a 70μm nylon mesh filter. After being counted, the cells were seeded into 6-well plates at a density of 10^5 cells/cm^2 (10^6 cells per well) in 3.5ml alphaMEM+10%FCS containing supplements to induce osteoblastic colony formation (100μM ascorbate-2-phosphate and 10nM dexamethasone). Each 6-well plate included the addition of one of the FGF-1 preparations (WT/CysFree or Chimera at 20ng/ml) +/- 5U/ml heparin.

The medium was replaced after 4 days, which removed any non-adherent cells, and thereafter every 3/4 days, each time the medium was replaced the growth factors and osteoblast-inducing factors were included. On the 18th day after seeding 10mMβ-glycerophosphate was added to the medium to allow mineralization to take place, and on the 21st day the colonies which had formed were fixed and stained for the presence of bone-related markers (alkaline phosphatase and mineralization).

The medium was removed from the wells, and PBS added to rinse the cells, this was removed and the colonies fixed in neutral buffered 10% formalin for 15 minutes at 4°C. The fixative was removed, and the colonies washed in dH_2O (1x1 min and 1x15 min). After removal of the second wash, AP substrate was added to each well (to 99.6 ml and 0.1M Tris-HCl pH8.3, add 10mg naphthol-AS-MX-phosphate dissolved in 400μl DMSO, mix well and add 40mg Fast Red Violot LB salt then filter to remove insoluble dye), and was incubated at room temperature for 40 minutes (an insoluble red dye will develop in the alkaline phosphatase positive colonies). After incubation, the substrate was removed and the colonies rinsed in tap water, this was followed by addition of Von Kossa's reagent to each well (2.5 silver nitrate in distilled water)

and incubation at room temperature for 30 minutes (mineralized areas of the colonies will turn black). The Von Kossa stain was then removed and the colonies rinsed twice in distilled water, this was followed by brief counterstaining with toluidine blue (0.1%w/v in 30%v/v ethanol) then two further rinses with distilled water, after which the stained colonies were allowed to dry. The results of one experiment are summarized in Figure 1. The figure shows the marrow cells after alkaline phosphatase (red) and Von Kossa staining for mineralized modules. The cells were treated with the indicated proteins in the presence or absence of 5U/ml heparin. Several points can be made from these studies. First, although heparin is known to potentiate the mitogenic activity of FGF-1, it clearly inhibits the formation of mineralized nodules in this assay. We assume the heparin inhibits the differentiation of the osteoblastic lineage from the marrow culture. The observation is particularly interesting given the large amount of literature on heparin induced osteoporosis. It is also clear that wild-type (WT) FGF-1 and the chimeric protein produce a significant increase in mineralized nodule formation whereas the cys free mutant of FGF-1 appears to inhibit. We showed previously that the cys-free mutant was ~10 fold more potent than wild-type in promoting mitogenesis of osteoblastic cells *in vitro* and producing new bone when injected locally over the calvaria. Although the cys-free is able to restore bone mass when given systemically, it does not appear to be 10 fold more potent in the osteoporosis model. This may be due to the increased mitogenic potency being off set by an inhibition of the formation of new osteoblastic cells from marrow stem cells. We believe the marrow culture is an important addition to our mutant analysis and that the differences seen among the different forms of FGF-1 is an important and exciting discovery.

Osteoblastic Migration and Attachment: We evaluated the ability of the chimeric FGF-1 to promote the migration and localization of human osteoblastic cells. The migration assays were performed using 24-well transwell filters (6mm diameter, 8μm pore size). Cells (MG-63) were trypsinized from stock plates, allowed to recover for 1 ½ hours in DMEM + 10% FCS prior to being seeded in the upper well at $1x10^5$ cells/cm^2 in DMEM. The lower wells contained wild-type FGF-1, FGF-1 chimera or BSA in DMEM. After 4 hours at 37°C in a CO^2 incubator the filters were removed, fixed in formalin and stained with 0.05% toluidine blue. The results of one such assay are shown in Figure 2. Panel A shows the BSA control. The arrow points to one of the pores in the filter. Panels B and C show the migration of cells through the filter in response

to FGF-1 (100ng/ml) and FGF-1 chimera (Panel C, 100ng/ml). In these panels the arrows point to migrated cells. Clearly the chimeric FGF-1 promotes increased migration of the osteoblastic cells. We also evaluated the chimeric protein as an attachment factor for osteoblastic cells. The chimera was pipetted in a line on tissue culture plastic, allowed to dry and washed. Osteoblastic cells were then added to the dish and allowed to attach overnight. Panel D of Figure 2 shows the boundary of the chimeric coating and reveals a tremendous increase in cell density on the coated portion (arrows point to boundary). Together these data demonstrate that the FGF-1 chimera is a potent chemoattractant and attachment factor for osteoblasts. It is not yet known whether the construct will target better to bone following systemic administration. It should effectively localize the growth factor to bone when applied locally.

Effects of Systemic Injection of Mutant Forms of FGF-1: Rats are ovariectomized, aged for 5 months to allow bone loss then started on daily tail vein injections of FGF-1 or modified forms for 28 days. The bone mass is allowed to stabilize for an additional 28 days without injections. The rats are then euthanized and the tibia, fibula and femurs are removed for histological analysis. In the past we demineralized tissue prior to sectioning and staining. This year we obtained an additional microtome (institutional funds) equipped with a tungstun/carbide knife that allows us to section mineralized bone. Tissue is cleaned from the bone then the bone is fixed in 70%, 95% and 100% ethanol for 48 hours each. The tissue is then infiltrated with plasticizer for two days then embedded at 37° C for 24 hours. 5 micron sections are cut and mineralized bone is visualized with Von Kossa silver stain. A summary of results is presented in Figure 3. The figure shows Von Kossa stained sections through the top of the tibia. Bone from a normal rat (NR1) and an untreated ovariectomized rat (OVX2) are shown at the top of the figure. The orange arrow points to well mineralized trabecular bone in the epiphyses (above growth slate) that is lost following ovariectomy. The orange arrows also point to regions in the treated animals (20μg/kg/day of wild-type, chimeric, cys free and the 678 mutated forms of FGF-1) where a dramatic restoration of mineralized bone mass was achieved. The green arrow point to regions where trabecular bone was clearly restored in the shaft. The tissues were subjected to quantitative analysis using the Bioquant Image Analysis system. The % mineralized tissue per high power field across the growth plate were; normal rat – 19.6%; ovx untreated – 5.4%; wild-type FGF-1 – 10.9%; chimeric FGF-1 – 13.7%; cys free FGF-1 – 14.8%; and the 678 mutant

20.5%. The key issue for the next year of support is to determine what combinations of these established forms can be produced and whether the multiple changes show enhanced anabolic activity for bone.

Effects of FGF-1 on Blood Pressure: Our last progress report was criticized for not having monitored the blood pressure effects of the various FGF-1 constructs. To some extent this was deliberate in that the studies are technically difficult and we have yet to settle on the most optimal mutant. We have begun these studies with a collaborator, Dr. Gregory Mundy. Phenobarbital sodium is used as an anesthetic and is given intraperitoneally as a bolus (50mg/kg), followed by intravenous infusion (88μg/min) in isotonic saline at 7μl/min. An IVC cannula is placed in the lateral cervical ventricle and a bladder catheter is implanted. Mean arterial blood pressure, systolic blood pressure, diastolic blood pressure and heart rate are determined following cannulation of the femora artery. The proteins are given as a bolus infusion. The results of two such studies are provided in Figures 4 and 5. At 30μg/kg both the wild-type and the cys-free mutant of FGF-1 resulted ~25% drop in systolic pressure (Figure 4). In contrast, at 5μg/kg the wild-type protein caused the same drop in pressure whereas the cys-free did not. These data demonstrate the concept of the original application is valid and the goals may be obtainable. We hope that future testing will be more routine. Towards this end we have used institutional funds to purchase a K18 5 cuff rat tail blood pressure system from Harvard Apparatus (~$5,000). The use of this system will result in reduced animal usage and we have sufficient baseline data to evaluate its performance in establishing the relative hypotensive effects of the various FGF-1 constructs.

Key Research Accomplishments

- Produced two new FGF-1 variants
- Established osteoblast migration and attachment assays
- Established stem cell differentiation assays
- Identified FGF-1 variants with enhanced anabolic activity for bone in osteoporosis model
- Established histological analysis of mineralized sections
- Demonstrated reduced hypotensive activity of cys-free mutant

Reportable Outcomes

- Tissue Engineering/Regenerative Healing/Stem Cell Biology Conference
  Cambridge Healthtech Institute, Pittsburgh, Pennsylvania
  Title: Fibroblast Growth Factor-1: Multiple Aspects of Bone Formation.
- D.J. Mackenzie, R. Sipe, D. Buck, W. Burgess, J. Hollinger. "Recombinant Human Acid Fibroblast Growth Factor and Fibrin Carrier Regenerate Bone." *J. Orthop. Res.* Submitted (1999).

Conclusions

Together, the data summarized in the body of this report and presented in previous progress reports demonstrate that the original hypothesis and statement of work were of merit and realistic. In the current progress report we summarize a large amount of work that could not have been anticipated in the original application due to technical limitations of the laboratory. We have identified three modifications of FGF-1 that show promise as improved anabolics for bone in a variety of *in vitro* and *in vivo* assays. The challenge for the final year of this application will be to identify the combinations of these modifications that result in the optimal growth factor for the rat osteoporosis model. Ultimately we must move the studies to a higher vertebrate species before we can consider studies in humans.

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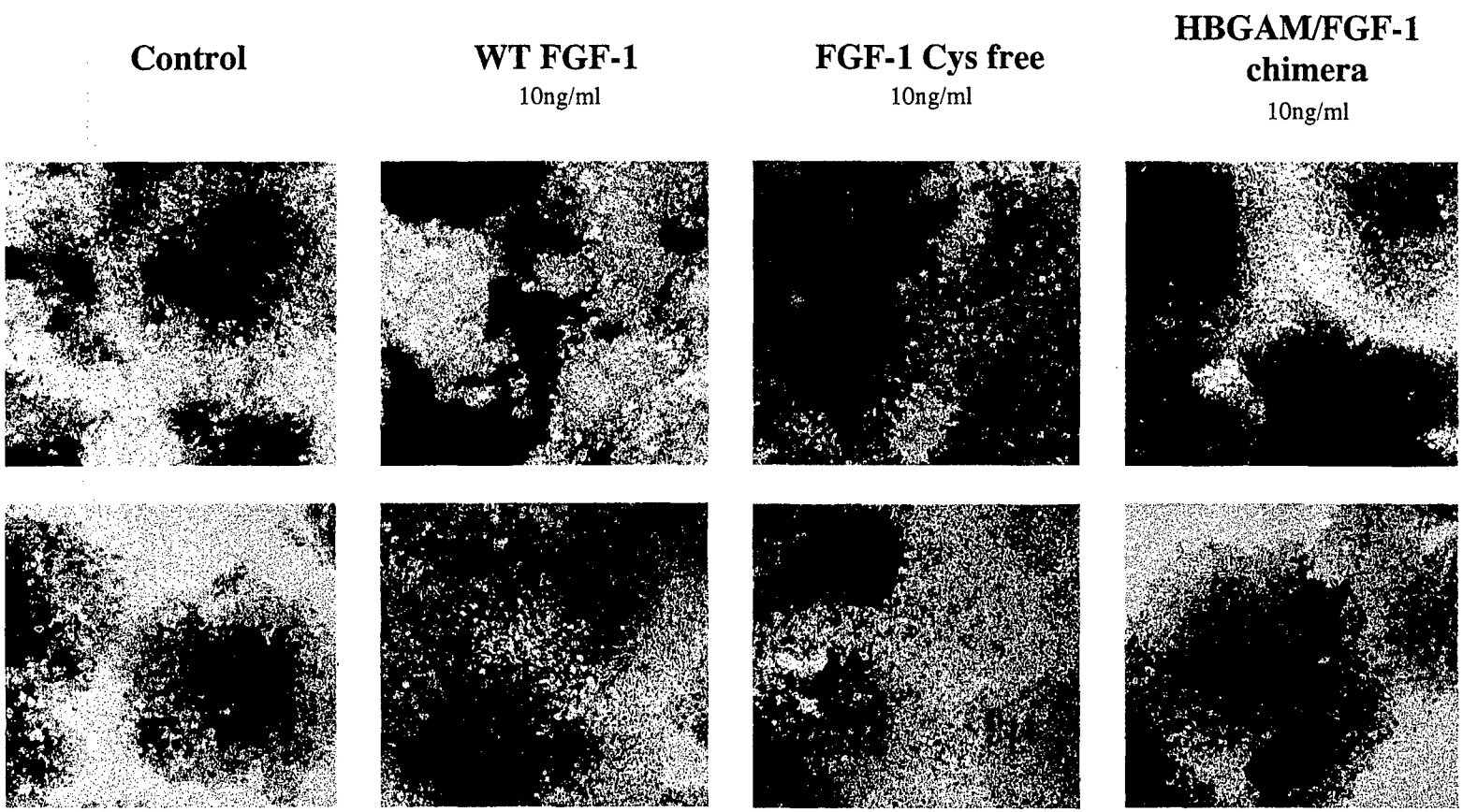
Rat marrow stromal cell CFU mineralization assay



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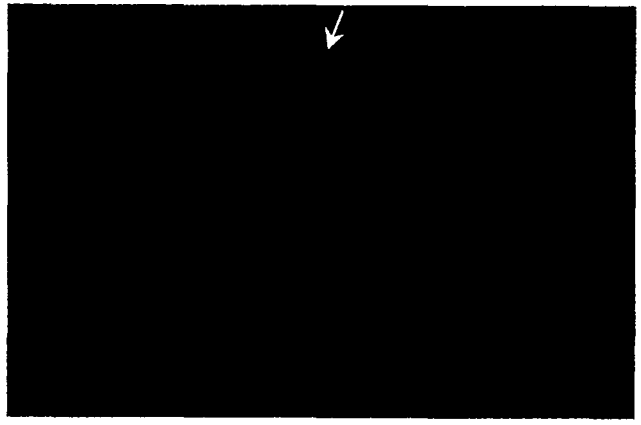

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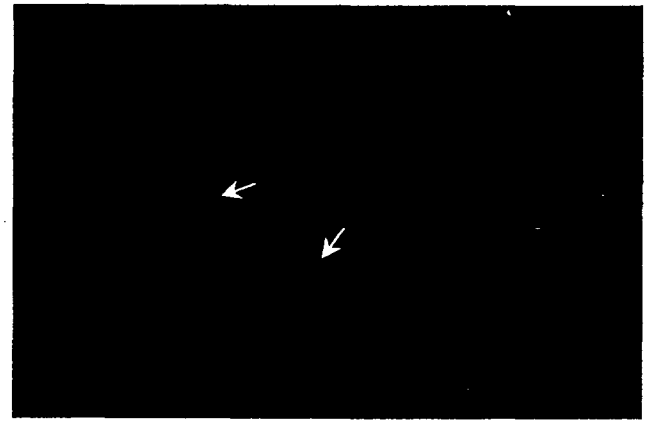

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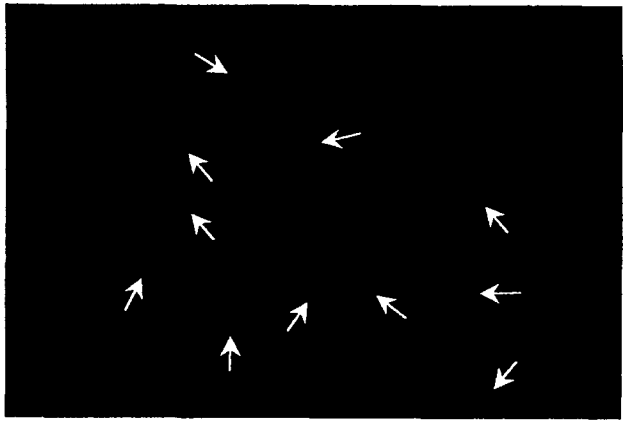

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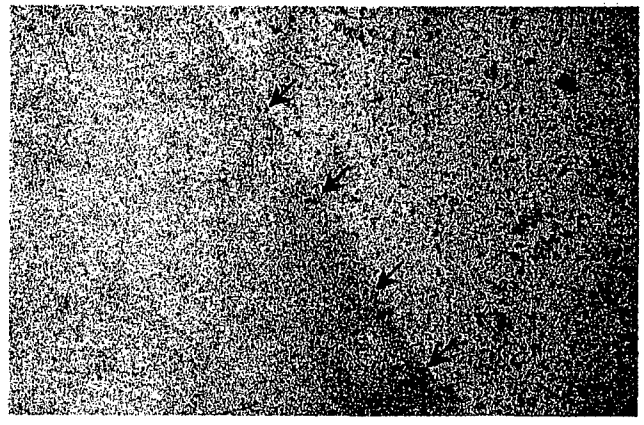

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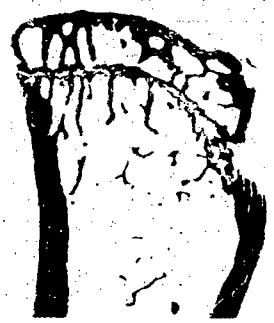

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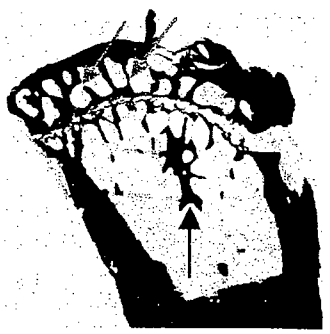

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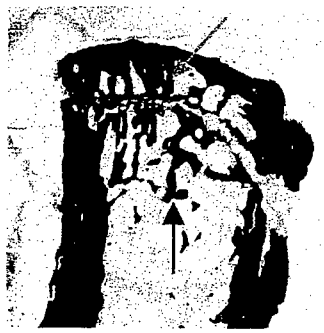
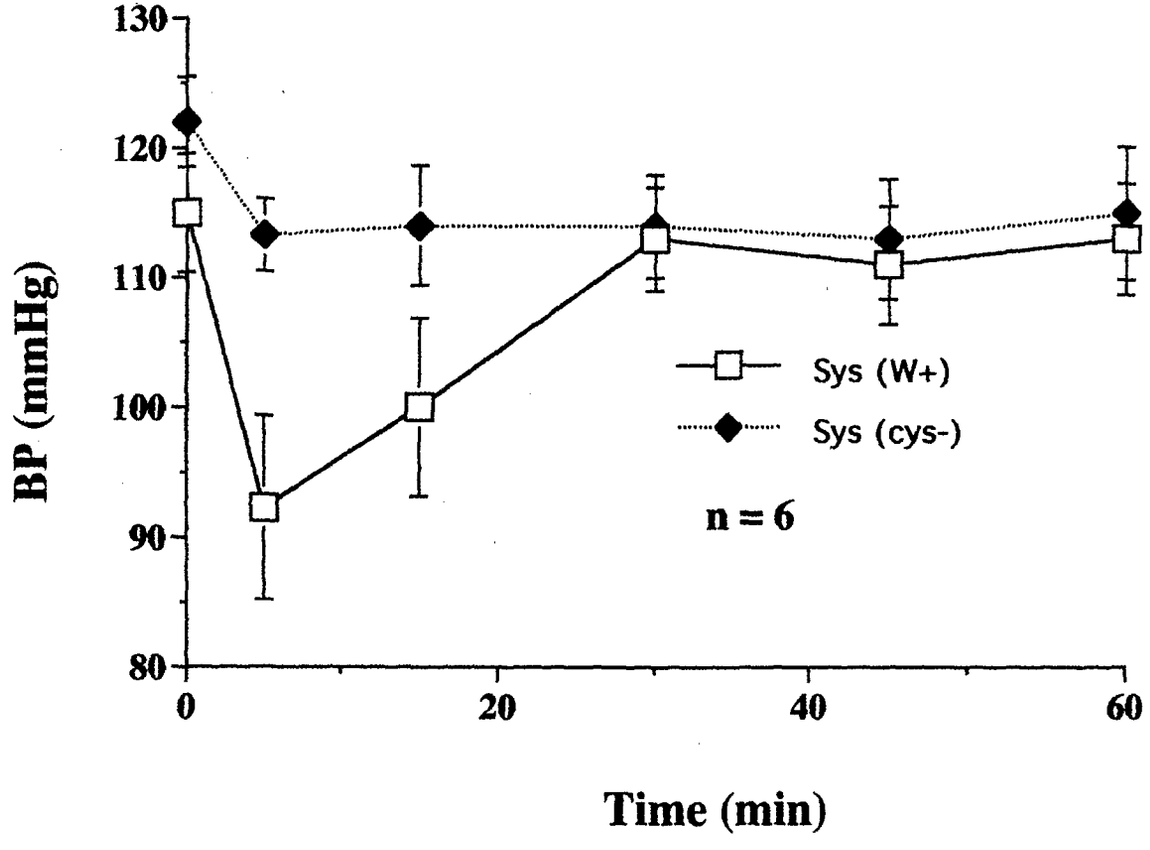
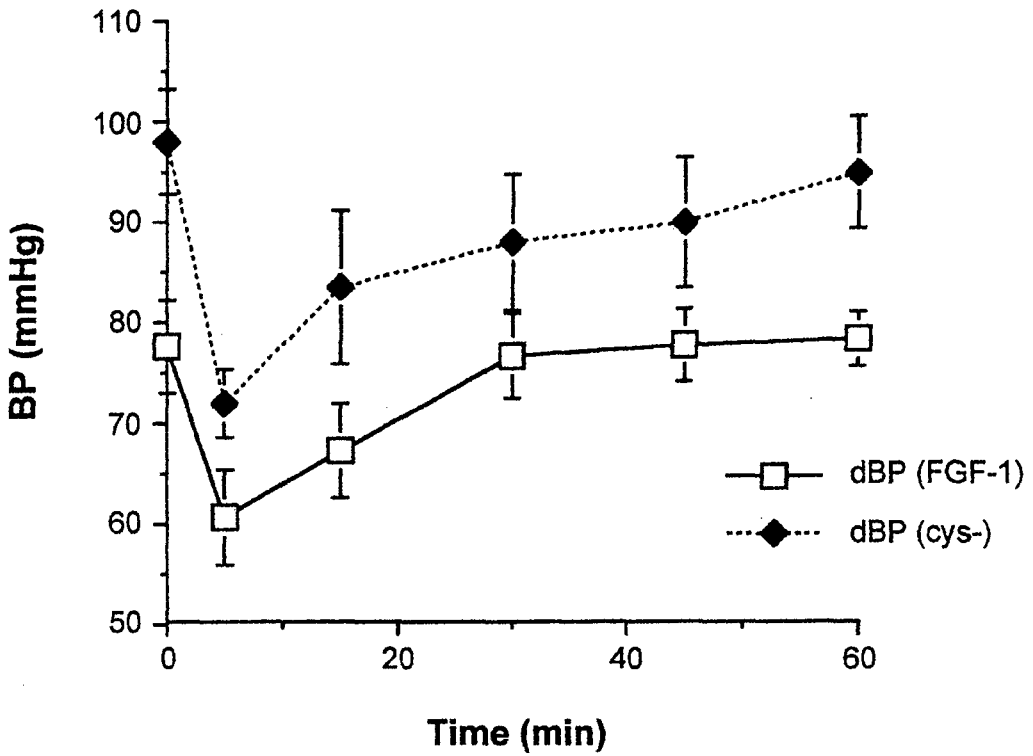


Effects of FGF-1 and Cys-Free Mutant on Systolic BP (5ug/kg)



Effects of FGF-1 and Cys-Free Mutant on Diastolic BP (30ug/kg)



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Appendix 4

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TITLE: Optimization of Fibroblast Growth Factor-1 as an Anabolic Agent for Osteoporosis

PRINCIPAL INVESTIGATOR: Mehrdad Tondravi, Ph.D.

CONTRACTING ORGANIZATION: American Red Cross
Rockville, Maryland 20855

REPORT DATE: October 2000

TYPE OF REPORT: Annual

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13. ABSTRACT ***(Maximum 200 Words)***

Osteoporosis is a disease that afflicts 200 million people worldwide, and that number is expected to increase significantly in the future. Currently, all approved osteoporosis drugs prevent bone loss by interfering with osteoclast function. The greatest therapeutic challenge in the field of osteoporosis is the identification of agents that promote significant bone formation. Fibroblast growth factor 1 (FGF-1) has been shown to be a potent bone anabolic peptide. The goal of this proposal has been to develop mutant forms of FGF-1 that maintain their bone anabolic potential while at the same time reducing its toxic effects (primarily epithelial hyperproliferation) upon systemic administration. Several mutant FGF proteins were developed and their bone anabolic potential compared. Among these Arg 136 » Lys mutant was the most osteoinductive followed by Cys-free > FGF-1 > FGF-HBGAM chimera. Furthermore, we demonstrate that injection of FGF-1 directly into the marrow cavity induces new bone formation suggesting the possibility of local delivery as a strategy to specifically increase the density of bone that are at risk of fracture.

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Introduction

This grant was originally awarded to Dr. Wilson Burgess and was transferred to me, due Dr. Burgess' departure from ARC, in June of 2000. The long-term goal of this research program remains to develop fibroblast growth factor-1 (FGF-1) via protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. Osteoporosis afflicts nearly 200 million people worldwide, and this number is expected to double in the next 20-30 years. It is likely that all people with the disease would benefit from treatments to increase bone mass. The greatest therapeutic challenge in the osteoporosis field at the present time is the identification of agents that promote significant new bone formation. Although there are effective resorption inhibitors for the treatment of osteoporosis (bisphosphanates, estrogens, and calcitonin), these drugs essentially stabilize bone mass but do not lead to substantial increases in bone mass or the restoration of trabecular bone microarchitecture. For patients with severe and established osteoporosis, there is a tremendous need for therapeutic agents that stimulate bone formation and initiate the cascade of events involved in osteoblast differentiation. Those agents that are known to have a stimulatory effect on new bone formation are fluoride, low-dose intermittent parathyroid hormone and its analogs, and the peptide growth factors that are incorporated into the bone matrix and released from bone as it is resorbed.

During the past several years, it has become apparent that members of the FGF family of growth factors and their receptors are essential for normal skeletal growth (1). The preliminary data that formed the basis of the original application demonstrated a significant osteogenic potential for local and systemic administration of FGF-1 *in vivo*. The data also documented certain toxicological or undesirable effects associated with these treatments. Together these data indicated that the therapeutic window is relatively narrow. In the progress report for the first year of funding, Dr. Burgess outlined his progress on the generation of several mutants of FGF-1 and chimeric proteins. He also reported on the *in vitro* and *in vivo* activities of these proteins. Those studies suggested that further evaluation of existing mutants and production of additional mutants or chimeric proteins may improve the efficacy of FGF-1 as an anabolic factor in the treatment of osteoporosis.

The original specific aims of the proposal were:

1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo* and to assess their toxicological or undesirable effects.
2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

In the following, I will summarize the progress that I have made towards these goals since June of 2000.

Body

The crippling effects of osteoporosis primarily manifest themselves in fractures of the femoral neck and vertebral bodies. Based on these clinical observations, we have begun testing the efficacy of various FGF constructs in local bone repair. Our objective is to increase bone mass by injection of a FGF preparation directly into bones that are at the highest risk of fracture. One advantage of this approach is that the required dose of FGF will be substantially less than that required for systemic administration, and therefore the undesirable effects of prolonged high dose systemic administration minimized. Importantly, since the end result would be increased synthesis in critical bones, for example femur, we believe that the clinical benefit will be substantial and potentially equivalent to systemic administration of FGF.

New FGF-1 Constructs: One new FGF-1 construct, Arg 136 » Lys FGF-1, was produced during the current year of support. The rationale behind this construct was as follows. We were interested in testing the bone anabolic efficacy of delivering FGF-1 locally by incorporating the growth factor in fibrin sealant (a mixture of fibrinogen and thrombin produced and marketed by the American Red Cross). We wished to minimize degradation of FGF-1 by thrombin, a protease that is required for clotting of fibrinogen and therefore a necessary component of the fibrin sealant delivery vehicle manufactured by the American Red Cross. Wild type FGF-1 is cleaved by thrombin at Arg 136, and ordinarily we add heparin to protect the FGF from thrombin cleavage. Although heparin renders FGF very resistant to thrombin cleavage, it inhibits mineralization by osteoblast cultures in vitro, and causes osteoporosis in vivo. For this reason, the thrombin cleavage site of FGF-1, Arg 136 was mutated to a Lys. In order to test if Arg 136 »

Lys mutant is more thrombin resistant 20 μg of it or the wild type FGF-1 were incubated with 6 units of thrombin for various times as indicate in Fig.1. The samples were then subjected to SDS-PAGE followed by coomassie blue staining, and the amount of intact vs. degraded FGF in each lane assessed. The data presented in Fig.1 clearly demonstrate that the Arg 136 » Lys mutant is significantly more resistant to thrombin than normal FGF-1.

Next we established that the Arg 136 » Lys FGF-1 maintains its mitogenic activity. In Fig.2, indicated amounts of FGF-1 or Arg136 » Lys FGF-1 were added to NIH3T3 cells for 22 hours. During the last four hours of incubation, 2 μCi of ^{3}H-thymidine was added to each well; subsequently the cells were harvested and the amount of label incorporated into DNA was quantitated. The results illustrate that the Arg 136 » Lys FGF-1 has identical mitogenic activity to the wild type protein.

Localized bone induction. The basic protocol has been to present FGF, or various mutant forms of it in a slow release form juxtaposed to calvarial bones for two weeks, followed by histologic assessment of bone formation. More specifically, we mix 1-5 μg of each FGF variant in 25 μl of water with 50 μl of 400 mg/ml fibrinogen, 24 μl of 5 U/ml thrombin, and 1 μl of 10,000 U/ml heparin in a 1 ml syringe. The fibrinogen / thrombin mixture carrier was developed by the American Red Cross and is marketed as ARC "Fibrin Sealant". Previous studies have shown that the fibrin sealant can be formulated to release trapped compounds, e.g. FGF-1, for up to 30 days. The fibrin / FGF-1 mixtures are incubated at room temperature for 2 hours to allow the fibrinogen to be cleaved by thrombin and form a firm gel. Mice are anesthetized with a mixture of xylazine and ketamine, the skin over the calvaria is opened and the preformed plug of fibrin sealant or fibrin sealant containing each FGF variant is placed over the calvaria. Subsequently, the skin is closed with wound clips. Two weeks later, the animals are sacrificed, the calvaria dissected and prepared for histology. Using this assay we compared the bone inductive affects of wild type FGF-1, Cys-free FGF-1, FGF-HBGAM chimera and Arg 136 » Lys-FGF. Figure 3 shows the amount of bone induced by each FGF variant. From these results we conclude that the Arg 136 » Lys mutant is the most osteoinductive followed by Cys-free > FGF-1 > FGF-HBGAM chimera. We presume that the Arg 136 » Lys mutant is more potent than the wild type FGF-1 due to its resistance to protease degradation.

A second series of experiments were designed to test the ability of FGF-1 to promote new bone formation directly in the intramedulary cavity. In this case, FGF-1 was mixed with fibrin sealant, as described above, except that the mixture was injected immediately, prior to the forming a gel, into the marrow space of anesthetized rat tibias. The injection needle entered the marrow directly through the skin and under the kneecap. We confirmed that the needle had entered the marrow cavity X-ray. Two weeks later the tibias of the rats were processed for histology. Figure 4 illustrates the presence of ectopic bone induced in the marrow as a response to FGF-1. This study illustrates the feasibility of administering FGF directly into bones that are at risk of fracturing.

Key Research Accomplishments

- Produced one new FGF-1 variant for a total of three FGF-1 variants since the beginning of this grant
- Established two new models for localized bone induction
- Established osteoblast migration and attachment assays (previous years)
- Established stem cell differentiation assays (previous years)
- Identified FGF-1 variants with enhanced anabolic activity for bone in osteoporosis model
- Established histological analysis of mineralized sections
- Demonstrated reduced hypotensive activity of cys-free mutant (previous years)

Reportable Outcomes

- Tissue Engineering/Regenerative Healing/Stem Cell Biology Conference
  Cambridge Healthtech Institute, Pittsburgh, Pennsylvania
  Title: Fibroblast Growth Factor-1: Multiple Aspects of Bone Formation.
- D.J. Mackenzie, R. Sipe, D. Buck, W. Burgess, J. Hollinger. "Recombinant Human Acid Fibroblast Growth Factor and Fibrin Carrier Regenerate Bone." *J. Orthop. Res.* Submitted (1999).

Conclusions

Together, the data summarized in the body of this report and presented in previous progress reports demonstrate that the original hypothesis and statement of work were of merit and realistic; although since taking responsibility of this grant in June, I have focused on localized

use of FGF-1. We have directly tested the bone anabolic effects of all currently available FGF-1 variants. The challenge for the final year of this application will be to identify the combinations of these modifications that result in the optimal growth factor for the rat osteoporosis model.

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1. Y. Li, K. Mangasarian, A. Mansukhani, C. Basilico. *Oncogene.* **14,** 1397 (1997).
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Effect of Thrombin Treatment On Wild Type and R136K Mutant FGF-1

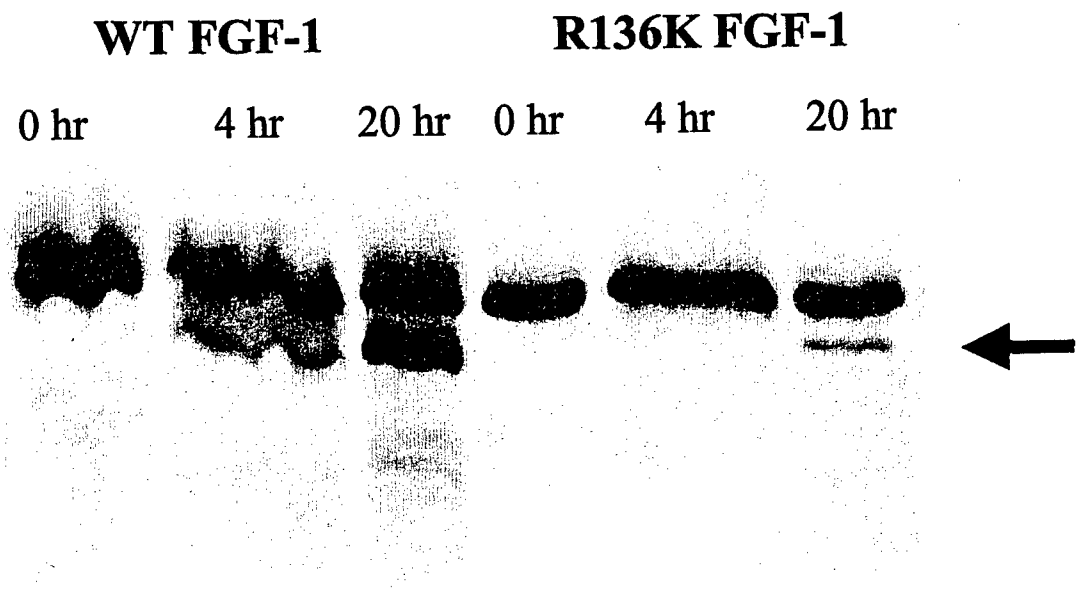


Fig. 1. The R136K mutation renders FGF-1 resistant to thrombin digestion. Twenty micrograms of wild type or R136K FGF-1 proteins were incubated at 37 °C with 6 U of thrombin in 600 µl reaction buffer (50 mM HEPES, 250 mM NaCl, 5 mM CaCl2). At indicated times, an aliquot was removed, boiled in sample buffer and ran on an SDS-PAGE follow by staining in coomassie blue. The lower band (arrow) represents the thrombin cleaved FGF. The relative intensity of the two bands in each lane, suggests that the R136K mutant is considerably more resistant to thrombin.

Mitogenic Activity of R136K FGF

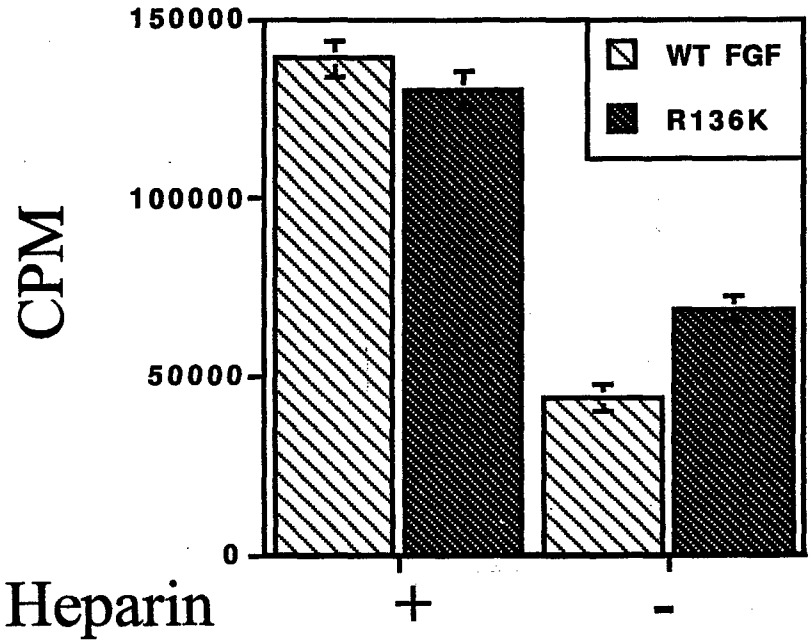


Fig. 2. The R136K mutant FGF-1 maitains its mitogenic activity. NIH3T3 cells were grown to 80% confluence, and transferred to medium containing 0.5% serum over night. Wild type FGF-1 or R136K mutant was added at 3 ng/ml without or with 10 U/ml heparin for 22 hrs. During the last 4 hrs of the incubation, 3H-thymidine was added at 0.5 μCi/ml. The cells were harvested and the amount of incorporated thymidine quantitated by scintilation counting. Results are from triplicates and error bars represent standard deviations.

Fibrin sealant carrier does not affect bone homeostasis

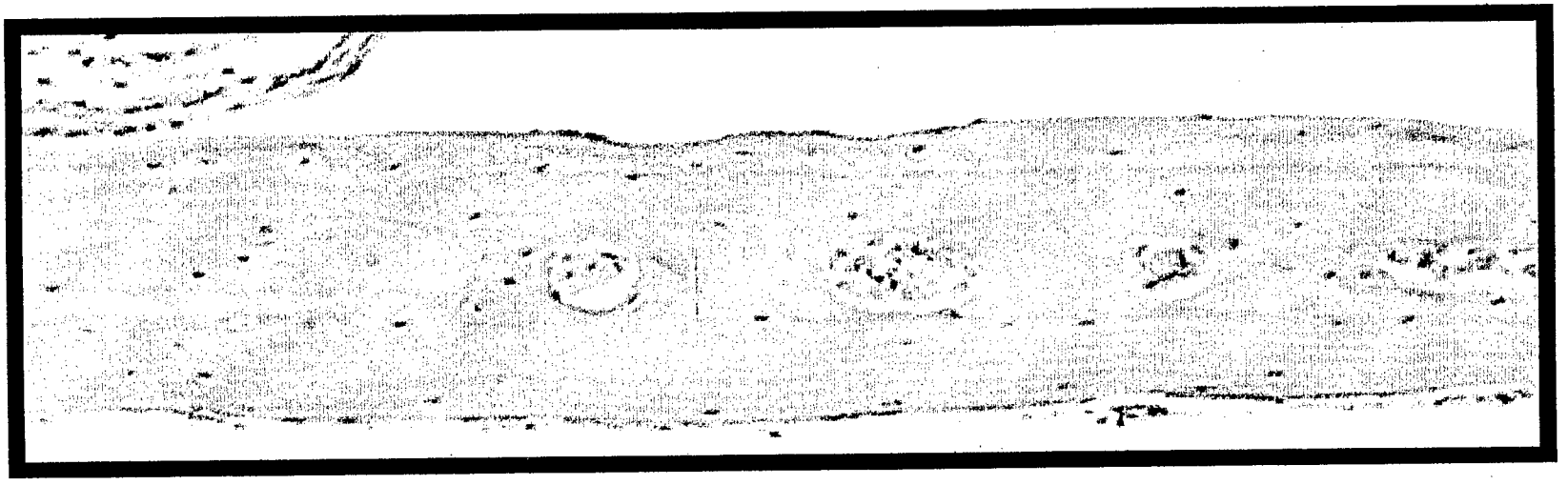
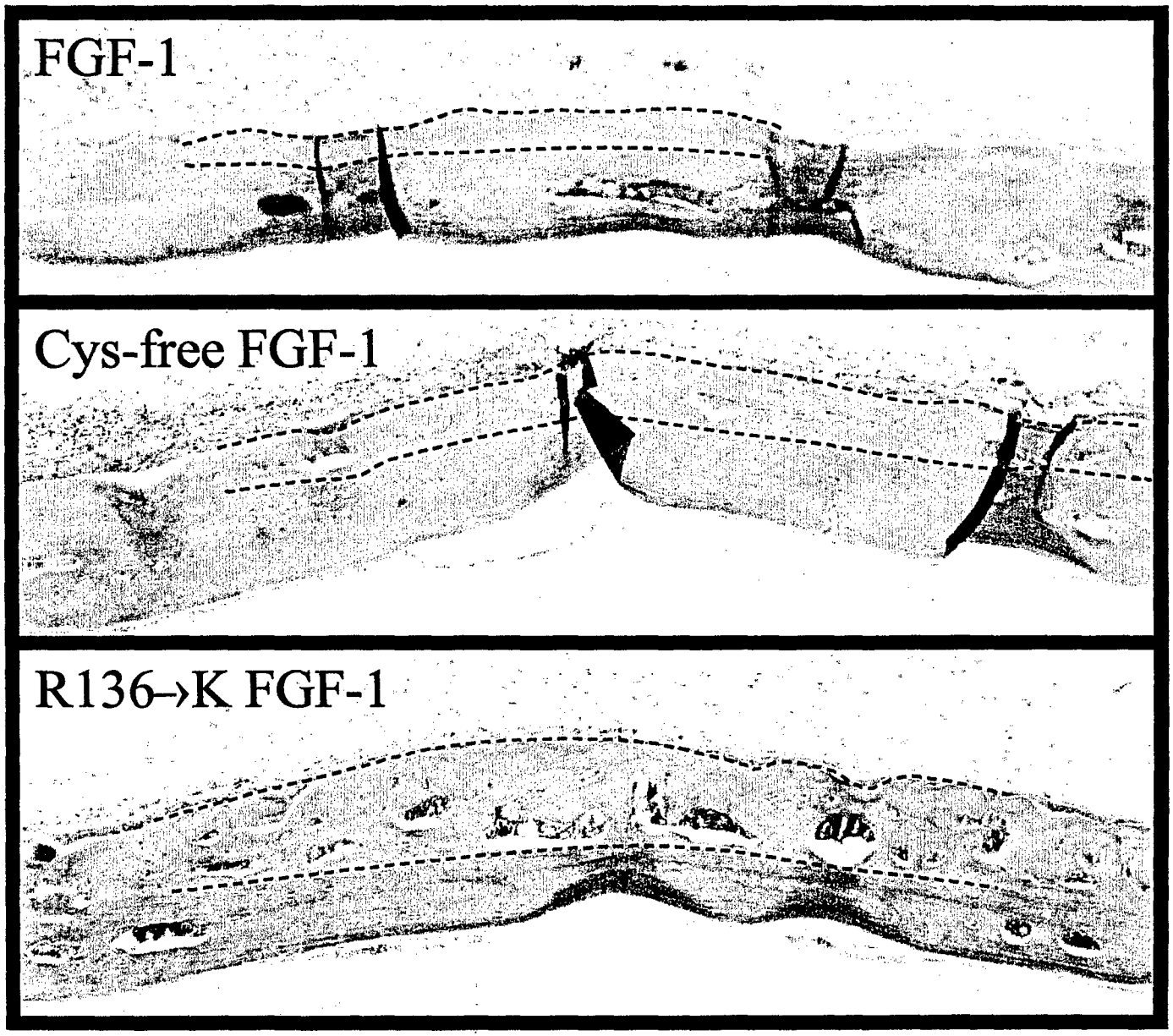


Fig.3a. Fibrin sealant carrier does not affect bone homeostasis. A plug of fibrin sealant consisting of 20 mg fibrinogen, 100 U thrombin, and 10 U heparin was placed on the calvaria of mice for 14 days. Subsequently the calvaria were dissected, fixed, decalcified, embedded in parafin, section and stained with H&E. This representative section illustrates that placement of fibrin sealant alone does not have any adverse affects on bone.

Bone induction by FGF-1 and its variants

Fig. 3b



Bone induction by FGF-1 and its variants

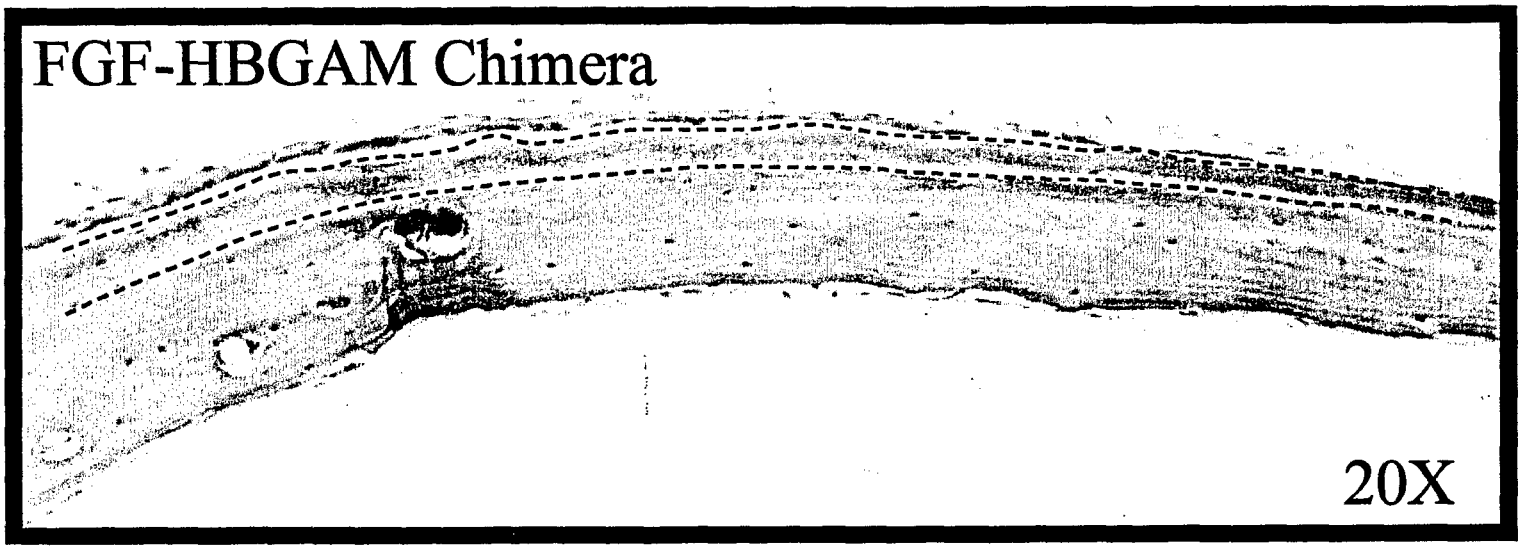


Fig. 3b. Bone induction by FGF-1 and its variants. A plug of fibrin sealant containing 5 μg of each FGF protein was placed on calvaria of mice as in Fig. 3a. Two weeks later the calvaria were examined histologically for evidence of new bone formation. In each panel the area of new bone is outlined by dotted lines.

DEPARTMENT OF THE ARMY
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PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl

ADB285846
ADB255323
ADB246514
ADB233740
ADB286395
ADB275662
ADB285859
ADB272667
ADB259790

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